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Antiviral signaling protein MITA acts as a tumor suppressor in breast cancer by regulating NF-κB induced cell death

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ABSTRACT

Emerging evidences suggest that chronic inflammation is one of the major causes of tumorigenesis. The role of inflammation in regulation of breast cancer progression is not well established. Recently Mediator of IRF3 Activation (MITA) protein has been identified that regulates NF-κB and IFN pathways. Role of MITA in the context of inflammation and cancer progression has not been investigated. In the current report, we studied the role of MITA in the regulation of cross talk between cell death and inflammation in breast cancer cells. The expression of MITA was significantly lower on in estrogen receptor (ER) positive breast cancer cells than ER negative cells. Similarly, it was significantly down regulated in tumor tissue as compared to the normal tissue. The overexpression of MITA in MCF-7 and T47D decreases the cell proliferation and increases the cell death by activation of caspases. MITA positively regulates NF-κB transcription factor, which is essential for MITA induced cell death. The activation of NF-κB induces TNF-α production which further sensitizes MITA induced cell death by activation of death receptor pathway through capsase-8. MITA expression decreases the colony forming units and migration ability of MCF-7 cells. Thus, our finding suggests that MITA acts as a tumor suppressor which is down regulated during tumorigenesis providing survival advantage to tumor cell.

1. Introduction

Breast cancer is the second most common form of cancer worldwide. About 1.3 million women are diagnosed with breast cancer annually and more than 400,000 women die from the disease around the world [1,2]. In spite of extensive efforts, there is significant morbidity and mortality associated; therefore, understanding the pathogenesis of breast cancer is of immense importance.

Evidences support the view that chronic inflammation contributes to initiation and progression of cancer [3–5]. The patients with ulcerative colitis and Crohn's disease are at increased risk for developing colorectal cancer. Similarly, inflammation and infection of liver are associated with increased risk of hepatic cancer [6,7]. The experimental evidences demonstrating association of inflammation and breast cancer are emerging. Chronic inflammation plays a critical role in breast cancer occurrence/recurrence [8]. Inflammatory Breast Cancer (IBC) is one of the most aggressive types of breast cancer. The symptoms of IBC like swelling, skin redness, and an orange peel like texture of the skin are similar to inflammation. IBC is often misdiagnosed as mastitis and even antibiotics are prescribed to the patients [9]. These observations suggest that there is a strong linkage between inflammation and breast cancer. The biochemical mechanisms regulating inflammation in breast tissue and their association with breast cancer are not understood.

NF-κB and IFNs are important cellular pathways associating inflammation and cancer. The regulation of NF-κB and IFN pathways is extensively studied; however, its modulation in stimulus specific manner and its significance to tumorigenesis are still not clear. Recent studies suggest that sub-cellular organelles, specifically mitochondria and ER, provide novel signaling platform for the assembly of signalosomes. Mitochondria are emerging as a central regulator of viruses and bacteria induced inflammatory pathways. The discovery of mitochondria associated viral signaling protein (MAVS) on the outer membrane of mitochondria and its role in regulating NF-κB and IFN pathway during viral and bacterial infections suggested a strong linkage between mitochondria and inflammation [10]. Similarly, ER associated protein MITA is another link that might help understand the linkage between ER, mitochondria and inflammation.

MITA plays an important role in inflammation through regulation of NF-κB and IFN [11]. MITA interacts with RIG-I, and MAVS associated signalosome. This further activates downstream kinase complexes: the 'non-canonical' IKK-related kinase TBK1 or IKK complex [12]. The TBK1 complex induces the phosphorylation and dimerization of the transcription factors (IRF3 and IRF7), which translocate to the nucleus

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and bind to IFN-stimulated response elements (ISREs), thereby expressing type I IFN genes and downstream IFN-inducible genes [13]. On the other hand IKK complex activates NF-κB, subsequently promoting the expression of pro-inflammatory cytokines and other cell survival/death genes.

Given the strong linkages of inflammation and cancer, we hypothesize that MITA may be critical regulator of either cell survival or cell death, however, evidences are still lacking. We studied the expression of MITA in tumorous tissues of human breast cancer patients as well as in different breast cancer cell lines and investigated its role as a potential tumor suppressor. We observed that expression of MITA is predominant in extra-tumoral tissue whereas lower in tumorous tissue. MITA sensitizes the breast cancer cells to TNF-α induced cell death. MITA induced NF-κB is essential for cell death as well as clonogenic ability of the cells.

2. Materials and methods

2.1. Cells and cell culture

MCF7, T47D and HBL100 breast cancer cell lines were obtained from National Center for Cell Sciences, Pune, India. MDA-MB-231 was a gift of Prof. R. P. Singh (Central University of Gujarat, India). MCF-7, ZR-75-1 and T47D cells were cultured in RPMI 1640 (Life Technologies, USA), HBL100 in Dulbecco's modified Eagle's medium (Life Technologies, USA) and MDA-MB-231 in Leibovitz's L-15 media (HI-MEDIA, India). The media used were supplemented with 10% FBS (Life Technologies, USA) and 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Life Technologies, USA). Cells were incubated at 37 °C, 5% CO2 in specified media. MCF 10A cells were cultured in DMEM F12 (INVITROGEN) base media supplemented with (10% horse serum) along with the following supplements: 1) cholera toxin (100 ng/ml), 2) EGF (20 ng/ml), 3) hydrocortisone (500 ng/ml), and 4) insulin (cell culture tested) (10 μg/ml).

2.2. Plasmids and reagents

MITA cloned in pCMV6-ENTRY plasmid was a gift from Dr. Hong-Bing Su (Wuhan University, China). p65shRNA (RelA1 shRNA and RelA2 shRNA) and control shRNA were provided by Dr. Edurne Berra Ramírez (Gene Silencing Platform, CICbioGUNE, Derio, Spain). MITA shRNA was a generous gift by Dr. Peter Chumakov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). $FDEVDG_2$ construct was gifted by Dr. Brian Seed (Department of Genetics, Harvard Medical School, Cambridge Street, Boston). Primary antibodies used were MITA (Proteintech, USA), caspase-8, PARP, p65 (Cell Signaling Technology, Inc., USA), β-Actin and GAPDH (Abcam, USA), IκBα (Cell Signaling Technology, Inc., USA). HRP-conjugated anti-rabbit and anti-mouse antibodies (Thermo Scientific, USA) were used. The reagents used were TNF-α (Tumor necrosis factor) (Biovision, USA), PDTC (Pyrrolidine dithiocarbamate) (Sigma Aldrich, USA) zVAD-fmk (N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone) (Biovision, USA), IETD-fmk (Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone) (Clontech, USA).

2.3. Transfection

MCF-7 cells were transfected using standard calcium phosphate method [14]. MCF-7 cells and T47D were transfected using X-treme gene transfect reagent (Roche, Germany). HBL100 cells were transfected with X-tremeGENE 9 DNA transfection reagent (Roche, Germany) as per manufacturer's protocol.

2.4. Collection of tissues

Human breast tumor specimens were obtained from patients undergoing surgery. Tissues were collected from the tumor zone (tissue within the tumor boundary), and normal zone (distal normal tissue at least 10 mm from the outer tumor boundary). A fraction of all tissues was fixed in formalin and embedded in paraffin for routine histopathological analysis. The rest of the fractions were frozen in liquid nitrogen and then stored at −80 °C for RNA and protein extraction. Ethical approval from institute's ethical committee was taken prior to collection of sample for each of the patients. Details of the tissue specimen used are given in Supplementary Table 1.

2.5. Immunohistochemistry

After de-paraffinization in xylene and hydration by gradient alcohol series, antigen retrieval was done by heat treatment in citrate buffer (10 mM, pH 6.0). The sections were incubated in 10% NSS (normal sheep serum) for 20 min to block non-specific binding and further incubated with antibodies against MITA (1:1000) in 0.1% BSA overnight at 4 °C. Sections were stained using Quick Universal ABC KIT (Vector) followed by peroxidase staining reaction with DAB/H_2O_2 as chromogen. The stained sections were observed under bright field light microscope (Nikon Eclipse 80i; Nikon Instech Co. Ltd., Kawasaki, Kanagawa).

2.6. Quantitative analysis of gene expression

Total RNA was isolated using Tri Reagent (Life technologies, USA) and was reverse transcribed to synthesize cDNA using Transcriptor First Strand cDNA synthesis kit (Roche, Germany) or SuperScript VILO cDNA Synthesis Kit (Life technologies, USA) according to the manufacturer's instructions. Real time PCR was performed using SYBR Premix Ex TaqTM (Takara, Japan) or SYBR mix (life technologies, USA) or Applied Biosystems as per manufacturer's instructions.

Specific primers of the genes are listed below.

1. MITA: Fwd 5′-CGCCTCATTGCCTACCAG-3′;
   Rev, 5′-ACATCGTGGAGGTACTGGG-3′;
2. TNF-α: Fwd 5′-CCCAGGGACCTCTCTCTAATCA-3′;
   Rev 5′-GCTACAGGCTTGTCACTCGG-3′;
3. β-Actin: Fwd 5′-TCGTGCGTGACATTAAGGGG-3′;
   Rev 5′-GTACTTGCGCTCAGGAGGAG-3′;
4. 16s rRNA: Fwd 5′-GAAACCAGACGAGCTACCTAAG-3′;
   Rev 5′-GCCTCTACCTATAAATCTTCCC-3′;
5. GAPDH: Fwd 5′-AGAAGGCTGGGGCTCATTTG-3′;
   Rev 5′AGGGGCCATCCACAGTCTTC 3′.

2.7. Western blot

Cells were plated at a density of 4.5×10^5 cells/well in the six well plate and transfected with indicated expression plasmid or shRNAs using calcium phosphate method. After 48 h of transfection, the cells were harvested, washed with ice cold PBS and lysed in buffer A (150 mM NaCl, 30 mM Tris–Cl, 10% Triton X-100, 10% Glycerol, 1× Protease Inhibitor (Roche, Germany). The equal protein was loaded and resolved on 11% SDS-PAGE. Protein was electroblotted on PVDF membrane at 110 V for 1 h at 4 °C. The membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) or 5% BSA (BSA (Sigma-Aldrich, USA), 0.1% Tween-20 in TBS-0.02 M Tris–Cl, 0.15 M NaCl) for 1 h at room temperature. The membrane was incubated overnight with specific primary antibody. After incubation, the membrane was washed three times with TBS-T (TBS containing 0.1% Tween-20) for 10 min and incubated with a secondary antibody at room temperature for 1 h. The membrane was washed three times with TBS-T and signal was visualized by using EZ-ECL chemiluminescence detection kit for HRP (Biological Industries, Israel) by exposing it to X-ray film.

For the western blotting from tissue samples, the tissue samples obtained from breast cancer patient were snap frozen in liquid nitrogen. The tissue was homogenized to fine powder in the presence of liquid

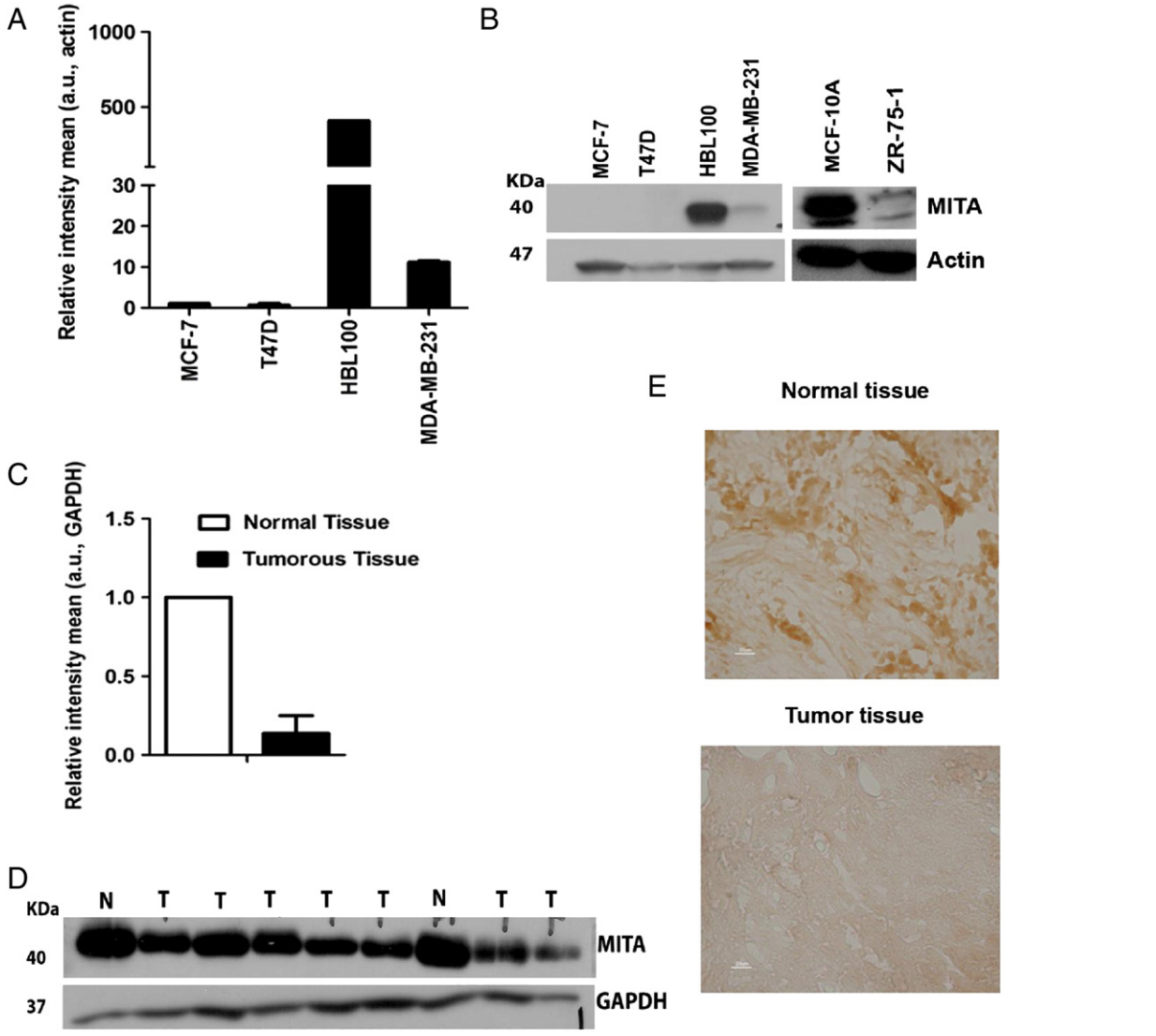

Fig. 1. Analysis of expression of MITA in breast cancer cell lines and different tumor tissue of breast cancer patient: (A) RNA was isolated from MCF7, T47D, HBL100 and MDAMB231 breast cancer cell lines, cDNA prepared and quantitative expression of MITA was analyzed using qPCR. (B) Protein level expression of MITA in different breast cancer cell lines was analyzed using western blot analysis using antibody against MITA. (C) RNA was isolated from tumorous and extra-tumoral tissues of breast cancer patients and relative expression of MITA was analyzed using qPCR. (D) Protein level expression of MITA was analyzed in the tumoral and extra tumoral tissue by western blot analysis using antibody against MITA. (E) Immunohistochemical analysis of tumoral and extra-tumoral tissues was done by incubating the tissue sections with antibody against MITA and detected using DAB staining.

nitrogen and lysed in RIPA lysis buffer(50 mM Tris [pH 7.4], 50 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.1% SDS, and 1% Triton X-100, 0.2% protease inhibitor cocktail, 1 mM PMSF, 2 mM NaF and 2.5 mM sodium pyrophosphate). The lysates were freeze thawed three times in liquid nitrogen. After 15 min of centrifugation (4000 rpm at 4 °C), the supernatant was saved to use as a whole-cell lysate. The protein was analyzed by western blotting as described above.

2.8. NF-κB luciferase assay

To assess NF-κB activity, MCF-7 cells were plated at density of 1×10^5 cells/well in 24 well plate and luciferase assay was performed as described previously using Dual-Glo luciferase assay system (Promega, USA) [15].

2.9. Caspase 3/7 and caspase-8 activity assay

The activity was performed using Caspase-Glo^R 3/7 Assay kit (Promega, USA) or Caspase-Glo^R 8 Assay kit (Promega, USA). Cells were plated at the density of 4×10^4 cells per well in 96 well white clear bottom plate and transfected with indicated expression plasmids or shRNAs and respective controls. Caspase-Glo^R 3/7 (10 μl) reagent or caspase-8 Glo reagent was added to each well and luminescence was measured with a Centro LB 960 Luminometer (Berthold Technologies, Germany).

2.10. Secreted GLUC activity assay for caspase activation in culture supernatant

Cells were plated in 24 well plate and co-transfected with MITA and a reporter construct FDEVDG2 [16]. The construct has a DEVD site placed in between GLUC reporter (*Gaussia* luciferase) and β-actin, so once the substrate site is cleaved by the caspases, luciferase will be secreted in the supernatant. After 24 h of transfection, the cells were treated with specific inducer of cell death. The supernatant (SN) was collected and centrifuged at 14,000 rpm for 5 min. Supernatant was diluted in 1:10 in 100 μl 1× lysis buffer. The substrate was added. 50 μl of substrate was added to 10 μl of this mixture and was analyzed with Centro LB 960 Luminometer (Berthold Technologies, Germany). Attached cells were lysed in 1× lysis buffer 100 μl per well for 15 min.

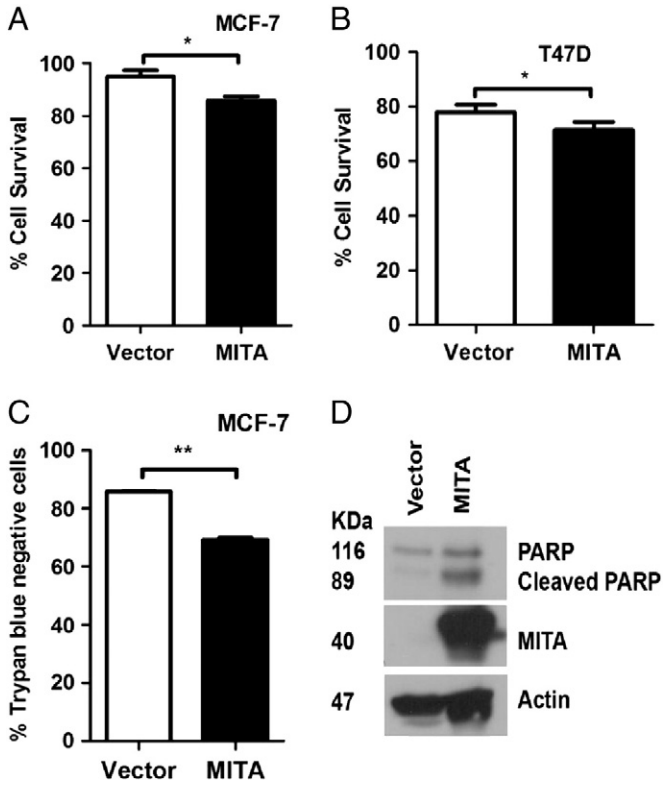

Fig. 2. MITA induces cell death: The specified cells were transfected with MITA and cell survival was monitored using (A and B) MTT reduction assay. Cell death was measured in MCF-7 cells by (C) trypan blue exclusion assay and (D) PARP cleavage.

10 μl of lysate was added to 50 μl of 1× substrate to detect that cellular GLUC activity luminescence was measured with a Luminometer. Total caspase activity was calculated in the SN as well as in cell lysate, and total caspase activity was calculated as the ratio of the caspase activity in SN versus (vs) cellular caspase activity.

2.11. Trypan blue exclusion assay

Cells were plated at the density of 1×10^5 cells/well in 24 well plate and transfected with the specific constructs. After 24 h of transfection, the cells were treated with TNF-α (10 ng/ml) for 24 h and stained with trypan blue. Minimum 100 cells per view were counted and percentage of cell survival was plotted.

2.12. MTT assay

The cellular proliferation was analyzed by MTT assay. MCF7 cells were plated in 24-well plate at a density of 1×10^5 cells/well. The cells were transfected with MITA as well as vector. After 24 h of transfection, 20 μl of MTT solution (5 mg/ml) (Serva, Germany) was added to each well and incubated for 2 h. After incubation, 500 μl of solubilization buffer (2% w/v SDS, 18.5% w/v formaldehyde) was added to dissolve the precipitate of purple colored formazan and color intensity was monitored using colorimetric microplate reader (BioTek Instruments, Inc. USA) at 595 nm wavelength.

2.13. Colony formation assay and scratch assay

Clonogenic activity of cells and migration ability of cells were determined as described previously [15,17].

2.14. Statistical analysis

Data are shown as mean ± SEM for no. of times experiment was repeated. Comparisons of groups were performed using student t-test for repeated measurements to determine the levels of significance for each group. The experiments were performed minimum two times independently and $p < 0.05$ was considered as statistically significant. GraphPad Prism was used to perform all the statistical analysis.

3. Results

3.1. Expression analysis of MITA in different breast cancer cell lines and patient samples

To study the role of MITA in initiation and progression of breast cancer, we analyzed the expression of MITA in different breast cancer cell lines. Relative expression of MITA in four different breast cancer cell lines (MCF-7, T47-D, HBL100 and MDA-MB-231) was analyzed by quantitative Real Time PCR. The expression of MITA was significantly lower in MCF-7 and T47-D cell lines as compared to HBL100 and MDA-MB-231 cells (Fig. 1A). The protein levels of MITA were checked in the same set of cell lines as well as in MCF-10A (non-tumorigenic mammary epithelial cells) and ZR-75-1 (ER-positive) cell lines by western blotting. The intense band of 40 kDa band corresponding to MITA was observed in HBL-100 indicating the strong expression of MITA. Similarly the non-tumorigenic cell line MCF-10A showed high level of MITA expression (Fig. 1B). The lower level of MITA was also observed in MDA-MB-231 as compared to HBL100 whereas it remained undetected in MCF7 and T47D (Fig. 1B). Similarly, ER positive cell line ZR-75-1 showed low level expression of MITA.

Expression of MITA was further investigated in tumor tissues obtained from breast cancer patients using quantitative real time PCR. Interestingly, significantly low RNA levels of MITA were observed in all tumorous tissues as compared to the extra-tumoral tissue (Fig. 1C). Similarly, protein levels of MITA were also low in all tumorous tissue as compared to the extra-tumoral tissue of the same patient (Fig. 1D). The expression of MITA was also analyzed by immunohistochemistry. Intense staining of MITA observed in case of extra-tumoral tissue as compared to tumorous tissue confirmed our observations (Fig. 1E). These evidences suggest that MITA is primarily expressed at higher levels in extra-tumoral tissue and pre-malignant cell lines, whereas, it decreases significantly in tumorous tissue and malignant breast cancer cell lines.

3.2. MITA induces cell death in breast cancer cell lines

As the expression of MITA decreased in tumorous tissue from breast cancer patient as well as in malignant cell lines, we hypothesized that MITA may be a potential tumor suppressor either by regulating cell survival or cell death. MCF-7 cell line having relatively low expression of MITA was chosen for further experiments. MITA was overexpressed in MCF-7 and cell proliferation was monitored using MTT. The transfection of MITA in MCF-7 showed decreased cell survival as compared to vector transfected cells (Fig. 2A). To eliminate the cell line specific action, T47D cells were transfected with MITA and cell survival was monitored. Decrease in MTT reduction was observed in case of MITA expressing cells as compared to control indicating decrease in the cell survival (Fig. 2B).

To further confirm if MITA induced decreased proliferation is due to cell death, the effect of MITA expression on induction of cell death was analyzed by trypan blue exclusion assay. The expression of MITA in MCF-7 significantly decreased trypan blue negative cells as compared to control cells, indicating increased cell death (Fig. 2C). The mechanism of cell death was further investigated. PARP is an established marker of apoptosis as it is a target of executioner caspases and is cleaved during apoptosis [18,19]. MCF-7 cells were transfected with MITA and PARP cleavage was monitored after 24 h of transfection. The western blotting

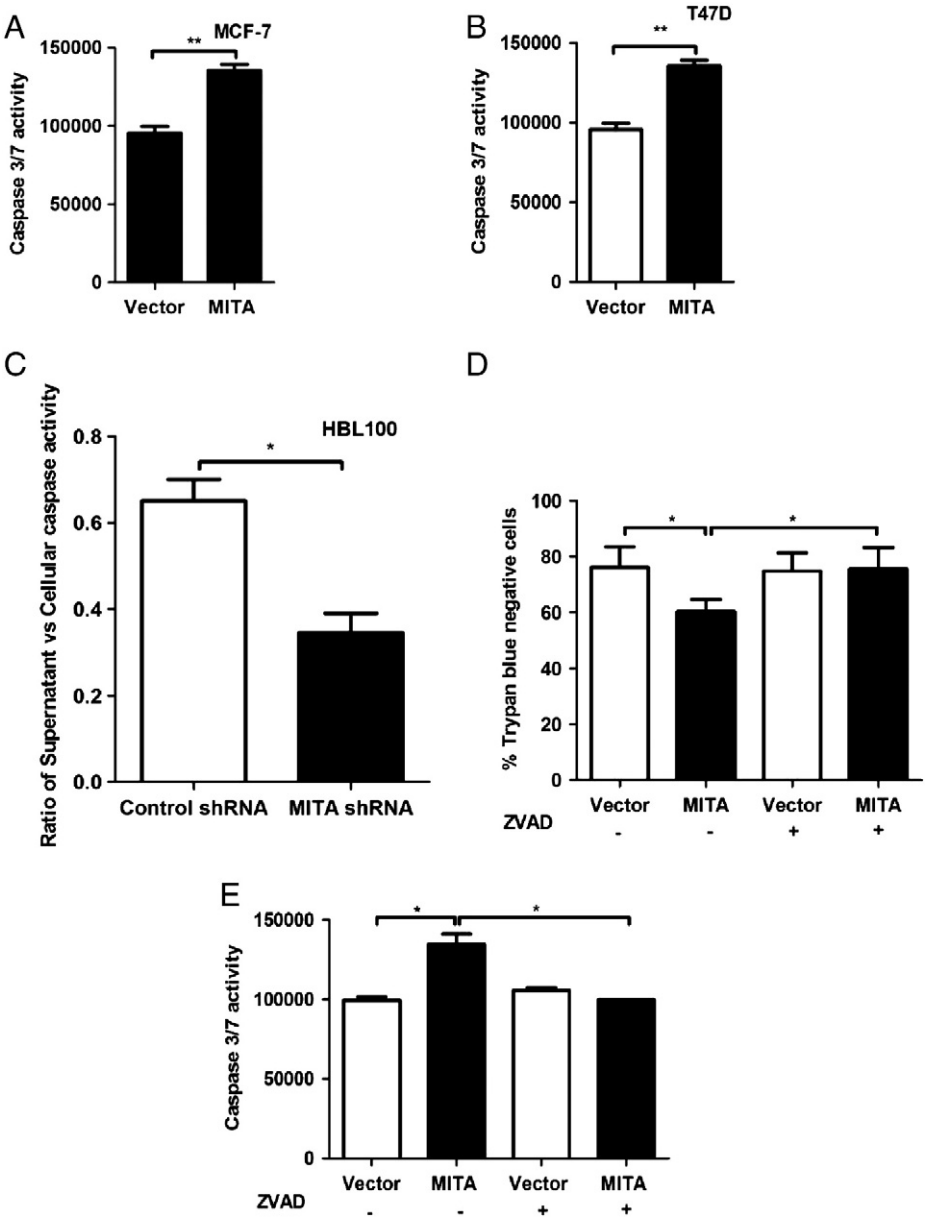

Fig. 3. MITA induces caspase activation during cell death: (A and B) The specified cells were transfected with MITA and caspase activity was measured using caspase glo substrate followed by luminescence measurement. (C) HBL100 cells were transfected with MITA shRNA along with the FDEVDG2 construct and the luminescence was measured in the SN and the cell lysate. Total caspase activity was calculated as the ratio of caspase activity in the SN vs cell lysate. (D and E) Caspases were inhibited in MITA transfected cells using PAN caspase inhibitor zVAD-fmk (20 μM) for 24 h. (D) Cell death was monitored using trypan blue exclusion assay and (E) caspase activity was measured using caspase glo substrate.

showed 110 kDa and 89 kDa band corresponding to native and cleaved form respectively. The expression of MITA showed increased levels of cleaved PARP (89 kDa) as compared to control (Fig. 2D).

The cleavage of PARP strongly suggests the activation of caspases in the presence of MITA. Caspases play a major role in initiation and execution of cell death; hence caspase 3/7 activation was analyzed using luciferase assay. The expression of MITA in MCF-7 significantly increased luminescence indicating enhanced caspase3/7 activity (Fig. 3A). The caspase activity was also monitored in T47D cell line in the presence of MITA and similar results were observed (Fig. 3B). As MITA was observed at higher level in ER negative HBL100 cells, MITA was knocked down in these cells to further confirm the role of MITA in cell death. The total caspase activity was monitored in HBL100 cell line using Gluc reporter based luciferase assay system in the MITA knocked down condition. The basal caspase activity decreased significantly upon MITA knocked down in HBL100 cells (Fig. 3C). The result further strengthened our hypothesis that MITA induces cell death by activating caspases. The role of caspases in cell death was further validated by inhibiting caspases using pan caspase inhibitor zVAD-fmk and then monitoring the cell death. The treatment of MITA transfected MCF-7 cells by zVAD-fmk significantly increased trypan blue negative cells as compared to control (Fig. 3D). Caspase activity was monitored by luciferase assay to confirm the inhibition of caspases. Decrease in caspase activities was observed in the cells treated with zVAD-fmk (Fig. 3E). These observations strongly suggest that inhibition of caspases rescues MITA induced cell death.

3.3. MITA regulates cell death by inducing NF-κB

NF-κB is a key regulator of pro- and anti-apoptotic genes during cell death. MITA is a key regulator of central inflammatory pathway [11], hence the role of NF-κB was analyzed in MITA induced cell death. MITA was co-transfected with NF-κB luciferase reporter construct in MCF-7 cells and luciferase activity was measured. The significant

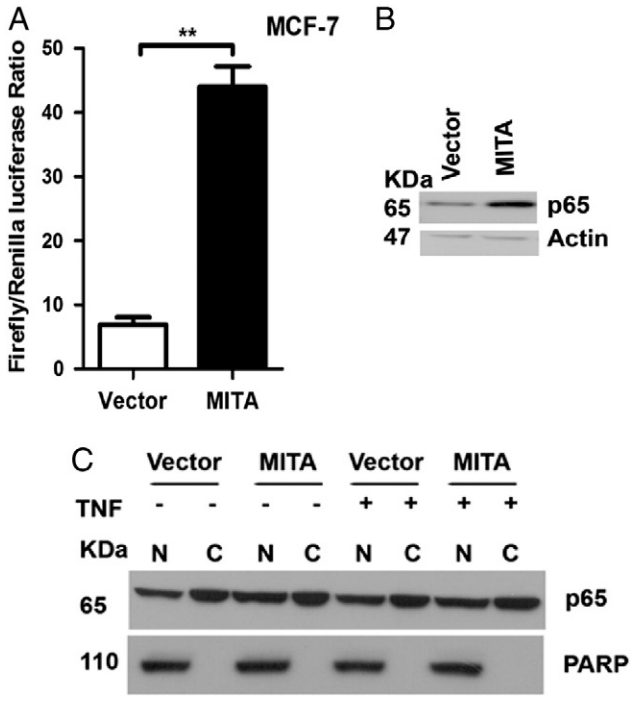

Fig. 4. MITA activates NF-κB transcription factor: (A) MITA was transfected in MCF-7 cells and NF-κB activity was measured using luciferase assay system. The activity was normalized and represented as Firefly/Renilla ratio. (B) MITA was transfected in MCF-7 cells and the level of p65 was analyzed by western blot using anti-p65 antibody. (C) Nuclear fraction and cytosolic fraction of MITA transfected cells were subjected to western blot analysis using p65 specific antibody.

increase in luciferase activity was observed in MITA transfected cells as compared to control indicating activation of NF-κB by MITA (Fig. 4A). The activated form of NF-κB is a heterodimer consisting of a p50 subunit and p65, and the expression of p65 is positively regulated by NF-κB [20]. Therefore we analyzed the expression of p65 in the presence of MITA by western blotting. Elevated level of p65 was observed in MITA expressing cells as compared to control (Fig. 4B). During NF-κB activation, p65/p50 translocates to the nucleus to execute its action. Therefore the translocation of NF-κB to the nucleus was analyzed by sub-cellular fractionation of MITA transfected cells along with the control. TNF-α was taken as a positive stimulus. We observed increase in the level of p65 in nuclear fraction of MITA transfected cells as compared to the vector in untreated as well as TNF condition (Fig. 4C). IKK complex is the central kinase complex during NF-κB activation [20]. Hence, to further understand the mechanism of NF-κB activation through MITA, components of IKK complex (IKKα, IKKβ, IKKγ) were knocked down using respective shRNAs in MITA transfected MCF-7 cells and NF-κB activity was measured using luminescence. p65, the most downstream component of NF-κB pathway was also knocked down using specific shRNA to analyze if MITA acts downstream of IKK complex. NF-κB activity was significantly suppressed upon any of the three components of IKK complex analyzed (Supplementary Fig. 1). The experimental evidences strongly suggest that MITA acts at IKK complex and activates NF-κB.

To understand the role of NF-κB activation in MITA induced cell death, NF-κB activation was inhibited using p65 shRNA as well as chemical inhibitor PDTC and cell death was monitored using trypan blue exclusion assay. The knockdown of p65 in MCF-7 significantly increased trypan blue negative cells in the presence of MITA as compared to control (Fig. 5A). Similar results were observed in case of chemical inhibition of NF-κB in MITA transfected cells (Fig. 5B). These evidences suggest that NF-κB activation is essential for cell death. We also confirmed the role of NF-κB in caspase activation by luciferase assay system. Increased caspase activity was observed in the presence of MITA; whereas knockdown of p65 using shRNA reverted back to the control (Fig. 5C). Similarly, the expression of MITA in MCF-7 cells treated with PDTC also showed no increase in caspase activity as compared to control (Fig. 5D). PARP cleavage was also monitored in similar conditions. The transfection of MITA showed increased levels of cleaved PARP (89 kDa) as compared to control. The knockdown of p65 in the presence of MITA showed decreased level of cleaved PARP as compared to control (Fig. 5E). These results convincingly demonstrate that MITA induced NF-κB is essential for the induction of cell death.

3.4. NF-κB regulates cell death by increasing TNF-α production

To further investigate the role of NF-κB in the regulation of MITA induced cell death, the expression of NF-κB regulated genes (BCL-XL, BCL-2, Bax, TNF-α, cIAP1, cIAP2 and XIAP) was screened in MITA transfected cells using quantitative PCR. Elevated expression of TNF-α was observed in the cells expressing MITA as compared to control (Fig. 6A); however no significant difference was observed in the expression of other genes (data not shown). The role of increased TNF-α and its contribution in regulation of MITA induced apoptosis were further investigated. MITA transfected cells were treated with TNF-α for 24 h and cell death was monitored. Significant decrease in the percentage of trypan blue negative cells was observed in TNF-α treated cells as compared to untreated cells in the presence of MITA indicating sensitization of MITA induced cell death by TNF-α (Fig. 6B).

Caspase-8 is a key player of TNF induced cell death therefore it was hypothesized earlier that caspase-8 activation may be an important regulator of cell death induced by MITA [21]. MCF-7 cells were transfected with MITA and caspase activation was monitored by western blotting. An intense band of 43/41 kDa corresponding to cleaved caspase-8 was clearly observed in MITA transfected cells as compared to control (Fig. 6C). To further confirm the role of caspase-8 in MITA induced cell death, caspase-8 was inhibited using a specific inhibitor IETD-fmk and cell death was monitored. Inhibition of caspase-8 increased the number of trypan blue negative cells in MITA expressing cells as compared to control (Fig. 6D). HBL100 cells showed high expression of MITA; hence we monitored the effect of knockdown of MITA on caspase-8 activity. The knockdown of MITA in HBL100 showed decreased caspase-8 activity (Fig. 6E). This indicates that caspase-8 plays a key role in MITA induced cell death. The above results showed that MITA expression activates NF-κB and induces the expression of TNF and that may initiate death receptor pathway. To confirm this p65 was downregulated by p65 shRNA in MCF-7 cells in the presence of MITA and caspase-8 activity was monitored. The caspase-8 activity was observed to be equivalent to control which was otherwise increased in case of MITA transfected MCF-7 cells (Fig. 6F). These evidences strongly suggest that endogenous expression of MITA may sensitize breast cancer cells to TNF induced cell death and its loss in tumor cells provided survival advantage.

3.5. MITA decreases clonogenic ability of MCF-7 cells

The experimental evidences in the current study showed that MITA is expressed at lower levels in breast tumor than normal cells. It also sensitizes MCF-7 to cell death. Therefore we hypothesized that it may be a potential tumor suppressor. This observation was confirmed by monitoring the clonogenic ability of the MFC-7 cells in the presence of MITA. The expression of MITA in MCF-7 cells significantly decreased colony forming units as compared to control (Fig. 7A). As we observed here that MITA induced NF-κB is responsible for cell death, the role of NF-κB in MITA induced reduction in clonogenic ability of the cells was also analyzed. MCF-7 cells were transfected with MITA along with control and p65 shRNA and clonogenic ability of the cells was monitored. The clonogenic ability of the cells significantly increased upon p65 knockdown in the presence of MITA as compared to control (Fig. 7B). These evidences strongly suggest that MITA decreases the clonogenic ability of MCF-7 cells by positively regulating NF-κB. We

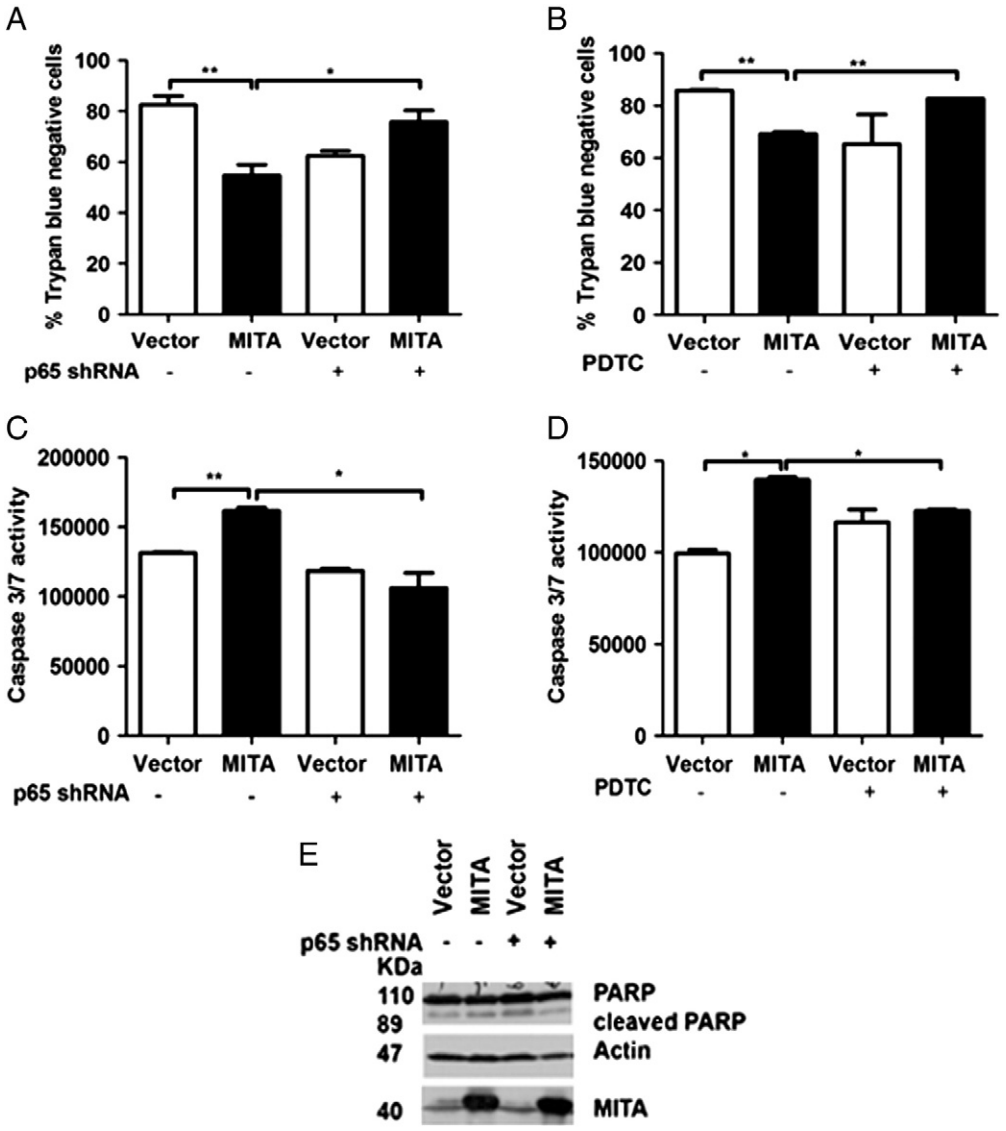

Fig. 5. MITA induced NF-κB is essential for cell death: MITA was transfected in MCF7 cells and NF-κB was inhibited using p65 shRNA or chemical inhibitor PDTC (100 μM) and cell death was quantified by (A and B) trypan blue exclusion assay. Caspase activity (C and D) and PARP cleavage were (E) also monitored in similar experimental conditions.

further checked the migration ability of MCF-7 cells. The cells were transfected with MITA and migration ability was analyzed using scratch assay. There was a significant increase in the open wound area of MITA transfected cells observed as compared to the control (Fig. 7C). To further confirm the role of MITA on migration ability of breast cancer cells, MITA was knocked down in HBL100 cells and its migration ability was monitored. There was a significant decrease observed in HBL100 cells upon MITA knock down (Fig. 7D). These observations strongly suggest that MITA regulated NF-kB negatively regulates clonogenic and migration ability of the breast cancer cells.

4. Discussion

The relation between inflammation and breast cancer is an emerging area. The current study is focused on ER resident MITA which is known to be essential for innate immune response against dsDNA virus and regulate both IFN and NF-κB pathway [11,22,23]. It has been observed that circulating tumor DNA levels increase in body fluids in different cancers including breast cancer [24–26]. Interestingly it had been observed that tumor DNA in complex with endogenous antimicrobial peptide LL37 can be transported back into endosomal compartments of plasmacytoid dendritic cells (pDC) leading to activation of type I IFNs [27]. It is possible that dsDNA induced pathway regulated by MITA may be linked to breast tumorigenesis which has not been investigated. In the current study, we demonstrated that MITA may be a potential tumor suppressor regulating NF-κB induced cell death in breast cancer.

The evidences in the current study suggest that expression of MITA is down regulated in tumor tissue as compared to normal. MITA is highly expressed in non-tumorigenic MCF10A cells whereas it is expressed at low levels in ER positive cells MCF-7, T47D and ZR-75-1. These findings suggest that during breast cancer progression, ER positive tumors specifically down regulate the proteins involved in innate immune response suggesting the evolved mechanisms to evade innate immune response pathways to facilitate tumor growth. This is further supported by loss of expression of RIG1, intracellular sensor of dsRNA, in ER positive cell lines [28]. It would be interesting to further study the correlation between the ER status and expression of MITA and other proteins involved in innate immune response and relevance during breast tumorigenesis.

The evidences in the current study clearly showed that MITA expression leads to cell death in breast cancer cell lines. The down regulation of MITA in tumor tissue and ER positive cell lines strongly suggests that it

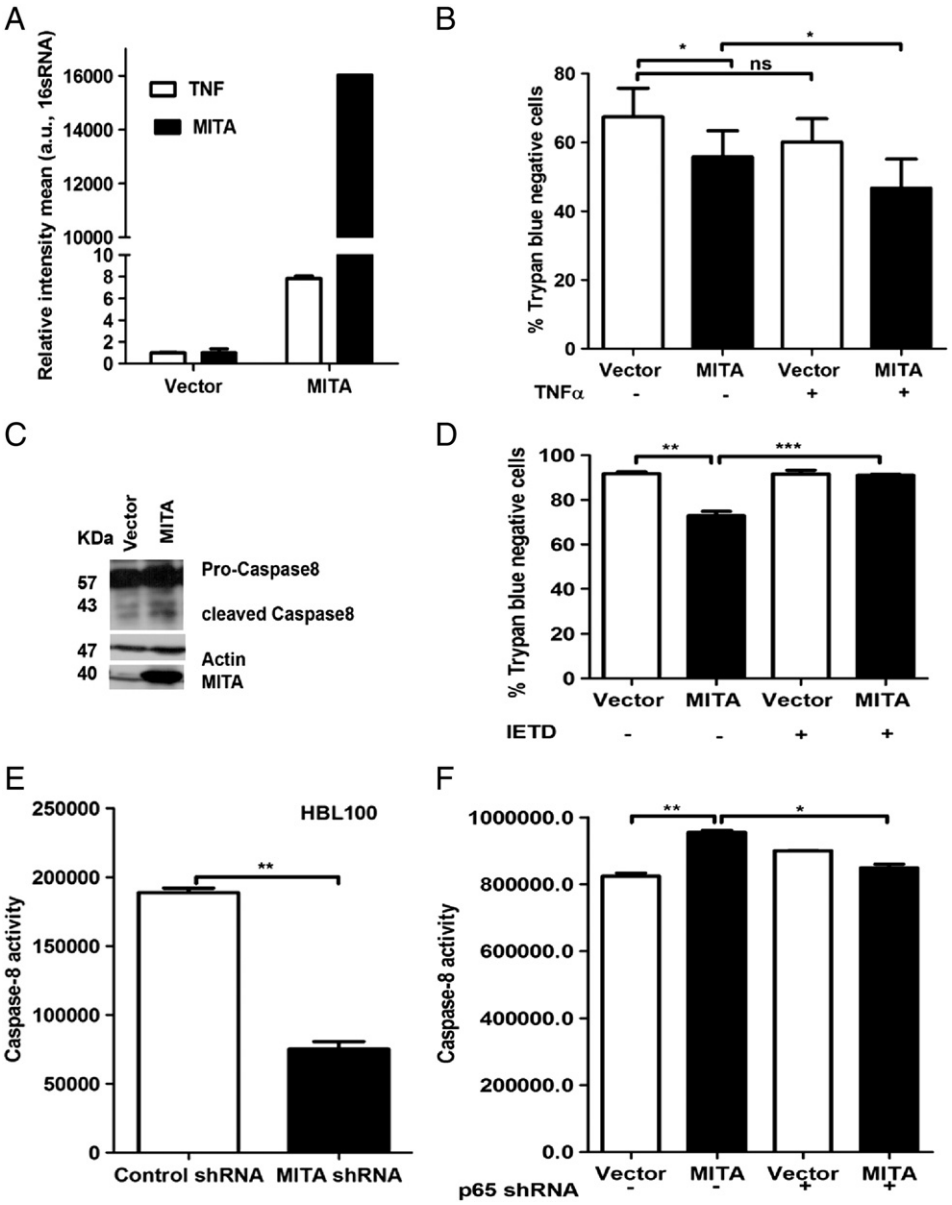

Fig. 6. MITA induced NF-κB sensitizes MCF-7 to TNF induced cell death: (A) MCF-7 cells were transfected with MITA. RNA was isolated and reverse transcribed to prepare cDNA. Relative mRNA level of TNF-α was quantified using real time PCR. (B) MITA transfected cells were treated with 10 ng/ml of TNF-α for 24 h and cell death was measured using trypan blue exclusion assay. (C) MCF-7 cells were transfected with MITA and caspase-8 activity was analyzed by using western blotting. (D) Caspase-8 activity was inhibited using IETD-fmk (1 μM) and cell death was monitored using trypan blue exclusion assay. (E) HBL100 cells were transfected with MITA shRNA and control random shRNA and caspase-8 activity was measured using caspase-8 glo substrate followed by luminescence measurement. (F) MITA was transfected in MCF7 cells and NF-κB was inhibited using p65 shRNA and caspase-8 activity was measured using luminescence.

may have important implication in regulating the cross talk of inflammatory and cell death pathway. As mentioned earlier, MITA is a critical regulator of NF-κB and IFN. These pathways are important cellular pathways associating inflammation and cancer [29,30]. The evidences here clearly demonstrated that MITA up regulates NF-κB pathway through IKK complex. Increased NF-κB activity has been found in both ER positive and ER negative breast cancer patients. The dysregulation of NF-κB and its implication to the breast cancer or any other cancer may be dependent on either loss or amplification of tumor suppressor or oncogene. The decreased expression of MITA and increased NF-κB activity provide advantage to the tumor cells. The association of NF-κB and breast cancer is further emphasized by recent observation of amplification of IKKε, a kinase regulating NF-κB pathway, in tumor tissue of breast cancer patients and breast cancer cell lines [31]. The gene is over expressed in over 30% of the breast carcinomas and provides survival advantage to tumor cells [31,32].

NF-κB is a dynamic transcription factor that induces the expression of several pro-apoptotic and anti-apoptotic genes. The role of NF-κB has been controversial as it may have pro-survival or apoptotic effect depending upon the stimulus and loss/gain of potential tumor suppressor/oncogene [33,34]. The current study also showed that MITA induced up regulation of NF-κB leads to high levels of TNF-α in breast cancer cells. Interestingly, TNF-α treatment further sensitized breast cancer cells to MITA induced cell death. TNF-α is known to bind to its receptor TNFR-I/II, either leading to NF-κB activation or cell death [35]. The p65 knockdown decreases the caspase-8 activity in MITA overexpressed conditions. The study strongly suggests that MITA induced NF-kB and increased level of TNF-α in breast cancer cells (MCF-7) lead to the activation of caspase-8 and downstream proteolytic cascade leading to cell death. The current study suggests that TNF-α shows antitumoric effect in the presence of MITA among its ability to play diverse role as pro or antitumoric agent. TNF in combination with melphalan is strongly

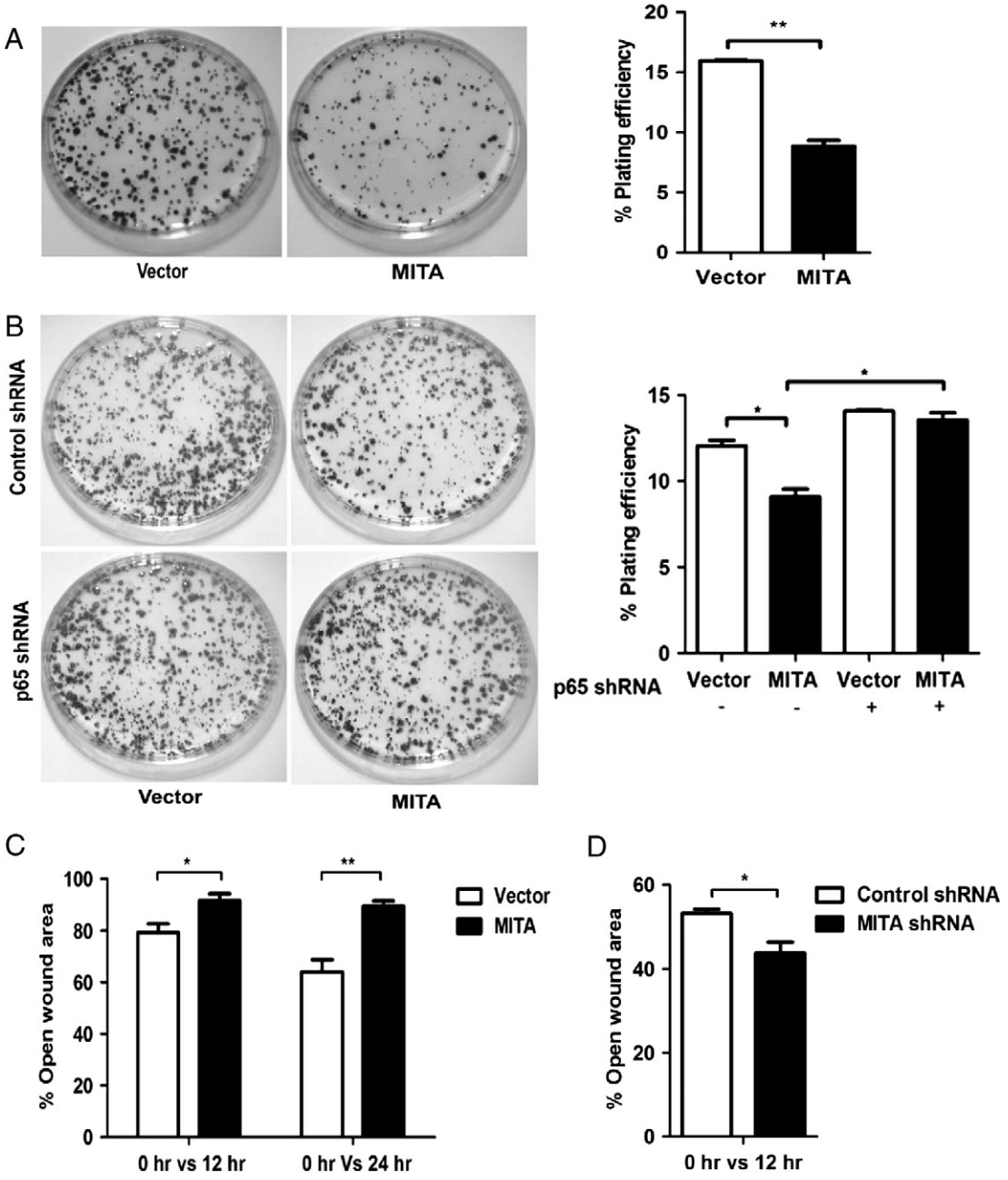

Fig. 7. The expression of MITA decreases clonogenic ability of MCF7 cells: (A) The cells were transfected with MITA and control vector or (B) co-transfected with p65shRNA or control random shRNA and clonogenic ability was analyzed as described in Materials and methods section. The colonies were stained with crystal violet for the assessment of clonogenic ability. (C) MITA was transfected in MCF-7 cells and migration ability of MCF-7 cells was checked by scratch assay as described in Materials and methods section. (D) MITA shRNA and control random shRNA were transfected in HBL100 cells and its migration ability was checked by scratch assay.

effective in the treatment of advanced soft tissue sarcoma [36]. Recently it has been shown that TNF-α expressing MDA-MB231 cells failed to form tumor *in vivo*. It also suggests that TNF-α interrupts symbiotic metabolic coupling between epithelial cancer cells and their host stromal microenvironment leading to death [37]. MITA down regulation in breast cancer tissue is a strategy of tumor to resist the antitumor effect of TNF-α.

The activation of cell death pathway strongly suggests that expression of MITA leads to decrease in the clonogenic ability. The migration ability of MCF-7 as well as HBL100 cells is also affected in the presence or absence of MITA respectively. The activation of caspase-8 is known to negatively regulate migration ability of the cells [38,39]. Caspase-8 binds to the lamella of the migrating cell and promotes the cell migration. Its catalytic activity is not required for the process. The decreased migration of MCF-7 cells upon MITA expression might be due to the increase in caspse-8 activity which ultimately makes pro-caspase-8 unavailable for binding and thus migration. Unraveling the mechanism of role of MITA in connecting these two observations is important to modulate the innate immune pathway for therapeutic intervention in breast cancer.

5. Conclusion

The current study provides strong evidences that MITA can act as potent tumor suppressor. MITA is significantly down regulated in breast cancer patients as well as in ER positive breast cancer cell line. The evidences in the current study suggest that MITA might prove to be an essential link to inflammation, endoplasmic reticulum and cancer. This

hypothesis clearly warrants further study to understand link between inflammation and breast cancer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.11.006.

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